Disposable solid state pH sensor based on flavin polymer-ferrocyanide redox couples

Disposable Solid State pH Sensor based on Flavin Polymer-Ferrocyanide Redox Couples.

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Abstract

An inexpensive and disposable screen printed sensor capable of monitoring pH is described. Based on a custom designed flavin derivative (10-(4-hydroxyphenyl) benzo[g]pteridine-2,4(3H,10H)-dione), electro-oxidation of the phenolic substituent leads to the deposition of a redox film whose peak positions are dependent on solution pH. The ability of the film to measure pH has been demonstrated and the possibility of using ferrocyanide as an internal standard has been critically assessed. The latter provides a Nernstian response and enables the production of carbon based electrode systems without the need for silver-silver chloride reference electrodes. The disposable electrode system has been characterised and shown to be robust with a potential drift (<4 mV) upon repetitive cycling.

Keywords

Flavin; Composite; Screen Printed Electrode; Sensor; pH

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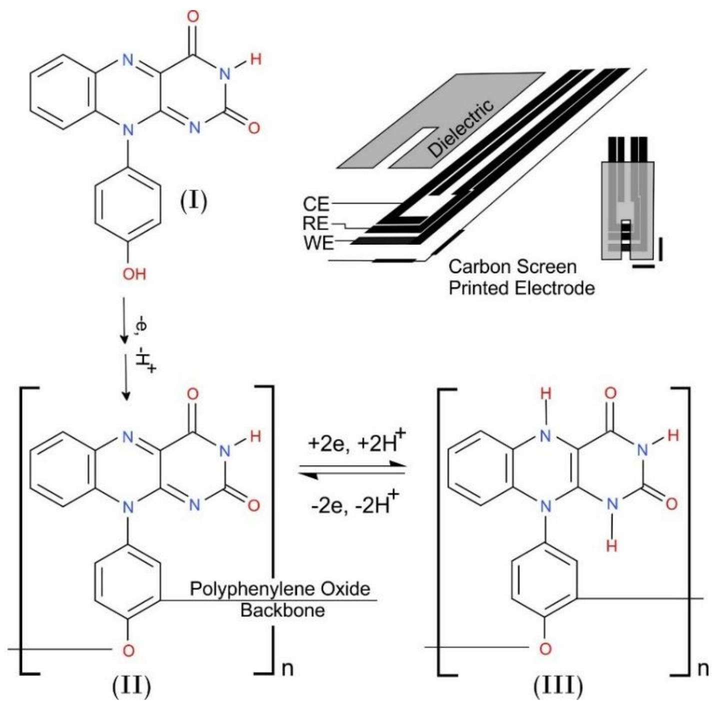
1.0 Introduction

The conventional glass membrane pH probe has long been the mainstay of laboratory pH measurements but new applications requiring miniaturised probes or disposability to counter issues of biological contamination has renewed interest in the development of new pH sensing systems. There is an extensive literature on the development of solid state potentiometric pH sensors with the exploitation of new nano material formulations based on MnO_2 [1], IrO_2 [2,3], WO_3 [4], NiO [5], RuO_2 [6] and ZnO [7]. Voltammetric approaches to the indirect measurement of pH have also gained ground in recent years as an alternative to the more traditional potentiometric systems [8–12]. These take advantage of the pH dependence of the oxidation peak potential of a particular marker compound with the corresponding shift in position (typically 59 mV/pH) being related to the pH of the test medium [9–11]. The pH marker compound can be a diffusing solution based species [8], immobilised as a single layer [12], polymer bound [9,11] or generated directly as an intrinsic species at the electrode surface [10]. Irrespective of the nature of the material employed, obtaining the necessary selectivity whilst providing a cost effective sensing strategy remains a considerable challenge. The aim of the present communication has been to investigate the pH sensing capabilities of a new flavin based marker and its integration within a disposable screen printed strip.

The electrochemistry of flavin compounds, particularly riboflavin, are well established and there has been substantial interest in their detection [13–16] but, more recently, they have found a niche as redox probes for a host of electroanalytical devices. They have been used as intercellular mediators in microbial systems [17,14,18], catalyst/mediators for a host of chemical and enzyme systems [19] and as probes for immunosensors [20,21]. In this work, a new flavin derivative bearing a phenolic substituent (**Scheme 1**) is electropolymerized on to the surface of a screen printed carbon electrode ($\mathbf{I} \rightarrow \mathbf{II}$) and exploited as a redox probe for the indirect measurement of pH. It was envisaged that the molecule would undergo two core transformations – oxidation and reduction of the flavin ($\mathbf{II} \rightarrow \mathbf{III}$ and $\mathbf{III} \rightarrow \mathbf{II}$) [21,22]. It was hoped that the characteristic redox transitions could be preserved post polymerisation and that the peak positions would shift with variations in pH in accordance with Nernst predictions.



Scheme 1. Electro-oxidation of the flavin monomer leading to the production of the a polyphenylene oxide chain with pendant redox groups ($\mathbf{I} \rightarrow \mathbf{II}$) and the subsequent redox transitions of the flavin unit ($\mathbf{II} \rightarrow \mathbf{III} \rightarrow \mathbf{II}$). Inset: Design of the screen printed sensor used throughout this work

There is however a clear limitation in this strategy in that the accuracy of the flavin peak positions, and hence the calculated pH, is dependent on being measured relative to a stable reference electrode. The addition of a solid state silver|silver chloride reference to the disposable strip is one option but, the potential of the latter will still be dependent on the activity of chloride ion within the sample matrix and, in principle, would be little better than having the carbon pseudo reference highlighted in **Scheme 1**. An alternative approach is to use a second redox probe which is insensitive to pH fluctuations to act as an internal standard – the peak shift of the flavin being measured relative to the internal standard. Lawrence and coworkers have demonstrated the use of copolymerisation of quinone-ferrocene hybrids as an elegant means through which to negate the vagaries of the silver reference [9,11,12]. The work reported here has sought to adopt a similar approach but exploit ferrocyanide as a much simpler (diffusible) and more accessible internal probe in the first instance as a means of assessing the analytical viability of the approach.

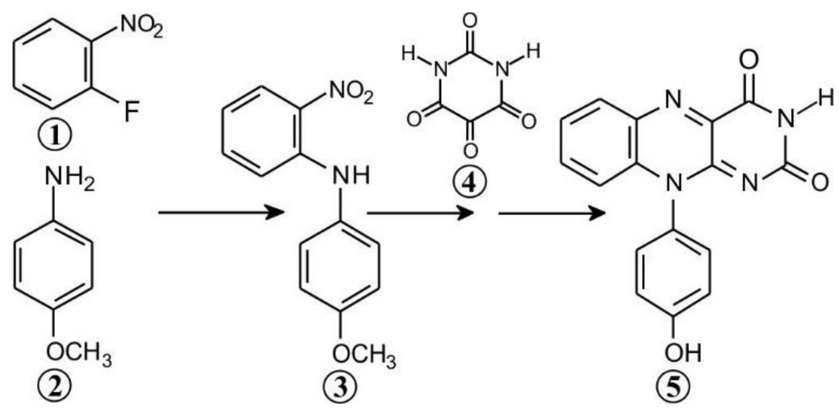
The electrochemical response of the immobilised flavin moieties under various pH regimes was investigated using a conventional Ag|AgCl (3M KCl) half cell reference and the performance compared with results obtained using an internal redox system. The robustness of the flavin component to repetitive/periodic monitoring was also assessed and the reversibility and potential drift through cycling through a series of pH regimes analysed. Soy milk products were used to assess the analytical accuracy of the system within a real matrix and to assess the signal integrity and potential interferences.

2.0 Experimental Details

Electrochemical Measurements: All chemicals were obtained from Sigma-Aldrich, were the highest grade available and were used without further purification. Electrochemical analysis was carried out using a micro Autolab Type III potentiostat with a standard three-electrode configuration (**Scheme 1**) with either a glassy carbon or screen printed electrode (0.32 mm^2 working area) as the working electrode (WE). A detailed schematic of the SPE design is included within the supplementary information. Platinum wire served as the counter electrode (CE) and a conventional silver/silver chloride (3 M KCl, BAS Technicol UK) reference electrode (RE). All measurements were conducted at 22°C ± 2°C.

Flavin Synthesis and Characterisation: A Bruker Avance-III 300 MHz spectrometer operating at ambient temperature was used for the collection of 1H and 13C data. Tetramethylsilane (TMS) was used as the internal standard for ^{1}H NMR with deuteriochloroform ($CDCl_3$, δC 77.23 ppm) and deuteriodimethylsulfoxide (d_6-DMSO, δC 39.51 ppm) used in ^{13}C NMR investigations. Chemical shifts are quoted in ppm and coupling constants in Hertz Hz using the high frequency positive convention. Electrospray ionization (ESI) mass spectra (Low and high resolution) was obtained with a hybrid linear ion trap-fourier transform mass spectrometer. Synthesis of the flavin (10-(4-hydroxyphenyl)benzo[g]pteridine-2,4(3H,10H)-dione) was achieved through the modification of existing methods [23–25]. The reaction pathway is summarised in **Scheme 2** and involves the initial reaction of p-anisidine (**1**) with 2-fluoro-1-nitrobenezne (**2**) in the presence of potassium carbonate to yield 4-methoxy-2-nitrodiphenylamine (**3**). The latter was isolated at the pump in 78% yield and the crude material (**3**) reduced using zinc dust under acidic conditions. The intermediate was subsequently treated with alloxan monohydrate (**4**) in the presence of boric acid to yield the 10-(4-methoxyphenyl)benzo[g]pteridine-2,4(3H,10H)-dione intermediate in 93% yield. This was demethylated using hydrobromic acid yielding the final derivative (**5**) in 98% yield.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.40 (s, 1H), 10.06 (s, 1H), 8.16 (dd, J = 8.2, 1.5 Hz, 1H), 7.75 (ddd, J = 8.7, 7.2, 1.6 Hz, 1H), 7.60 (ddd, J = 8.3, 7.2, 1.2 Hz, 1H), 7.19 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 8.8 Hz, 2H), 6.86 (dd, J = 8.6, 1.2 Hz, 1H). ^{13}C NMR (75 MHz, d_6-DMSO) δ 160.12, 158.78, 158.78, 156.12, 152.53, 139.88, 135.22, 135.15, 135.09, 131.78., 129.28, 127.43, 126.37, 117.05. IR (ATR, cm^{-1}): 3052.32, 3151.73, 1716.20, 1666.31, 1611.52, 1527.94, 1503.20, 1191.86, 875.96, 844.29. MS (ESI) m/z: 307.1 [M^+]. Melting point = >300°C.



Scheme 2. Synthetic pathway for the preparation of the flavin derivative

Electrode Fabrication: Screen printed electrodes (SPEs) were prepared using a Reprint International R23 Semi-Automatic Screen Printer. Carbon ink (Gwent Group C2030519P4) was printed onto AGFA Synaps® Offset Matt polyester substrate (450 g/m^2) through prepatterned stainless steel meshes (325 CL (50/30)) to generate the design outlined in Scheme 1. The electrodes were dried at 180°C prior to sectioning

Electrochemical Anodisation: Electrochemically anodising carbon electrodes (+2 V, 0.1 M NaOH) has been shown to improve electrochemical behaviour. It has been found that electro-oxidation increases exfoliation of the carbon particles bound within the screen printed film [8]. Similar work with carbon fibre substrates has demonstrated that such oxidation processes generate more edge plane sites and increases the populations of various oxygen functional groups (i.e. COOH, OH, C=O) [10].

Flavin Electropolymerisation: Polymerisation was achieved through placing the screen printed into an aqueous solution containing the Flavin phenol (**I**) derivative. The polymerisation solution consisted of the flavin (150 μM) dissolved in Britton Robinson buffer (pH 7) with 0.1 M KCl as the supporting electrolyte. The solutions were degassed with nitrogen during prior to commencing the polymerisation cycles and the latter run under quiescent conditions with nitrogen blanketing the cell. Repetitive scan cyclic voltammetry (+0.2 V $\rightarrow$ -0.8 V $\rightarrow$ +1 V, 50 mV/s) was used to initiate the electropolymerisation process. The magnitude of the final peak processes attributed to the flavin is dependent on the number of polymerisations scans employed. In the present instance, it was found that 3-5 polymerisations scans gave rise to well defined redox peak processes in the final film and this approach was used throughout.

3.0 Results and Discussion

Cyclic voltammograms detailing the response of the screen printed electrode to the flavin compound (150 μM, pH 7, 0.1 M KCl) are detailed in **Figure 1**. The potential was initially scanned from +0.2V to -0.8 V whereby the reduction of the flavin ($\mathrm{I} \rightarrow \mathrm{II}$) can be observed at -0.408 V. The corresponding oxidation was observed at +0.336 V (ΔE_p = 72 mV) and the oxidation of the phenolic component appears as a broad, irreversible peak at +0.82 V. The most notable feature from the voltammograms detailed in **Figure 1** is that the flavin redox peaks appear to increase with each scan indicating the that material is aggregating at the electrode surface. This is only observed when the potential is swept to sufficiently positive potentials whereby oxidation of the phenolic substituent occurs and polymerisation is induced.

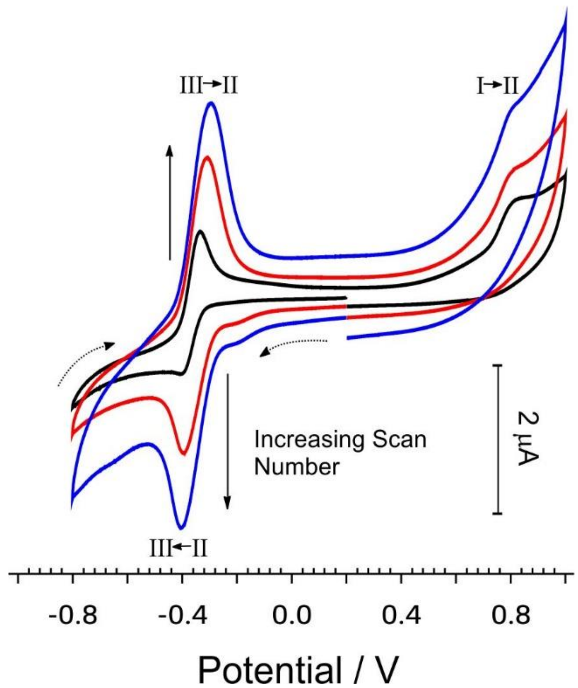


Figure 1. Cyclic voltammograms detailing the response of a screen printed carbon electrode towards the flavin derivative in pH 7 buffer with 0.1 M KCl. Scan rate: 50mV/s

The influence of pH on the electrode response was assessed through using square wave voltammetry to enable a more accurate rendering of the peak positions. While the voltammograms detailed in **Figure 1** were run under nitrogen to enable clear resolution of the reduction peak processes, it would be impractical to do so in a decentralised context – particularly where the emphasis is on a simple, disposable sensor. As such, the square wave voltammograms used throughout the following discussions were recorded in air. The oxidation peak was chosen on the basis that interference from the reduction of oxygen would be minimal on the forward (anodic) sweep. Square wave voltammograms detailing the response of the flavin modified SPE under various pH regimes are compared in **Figure 2**.

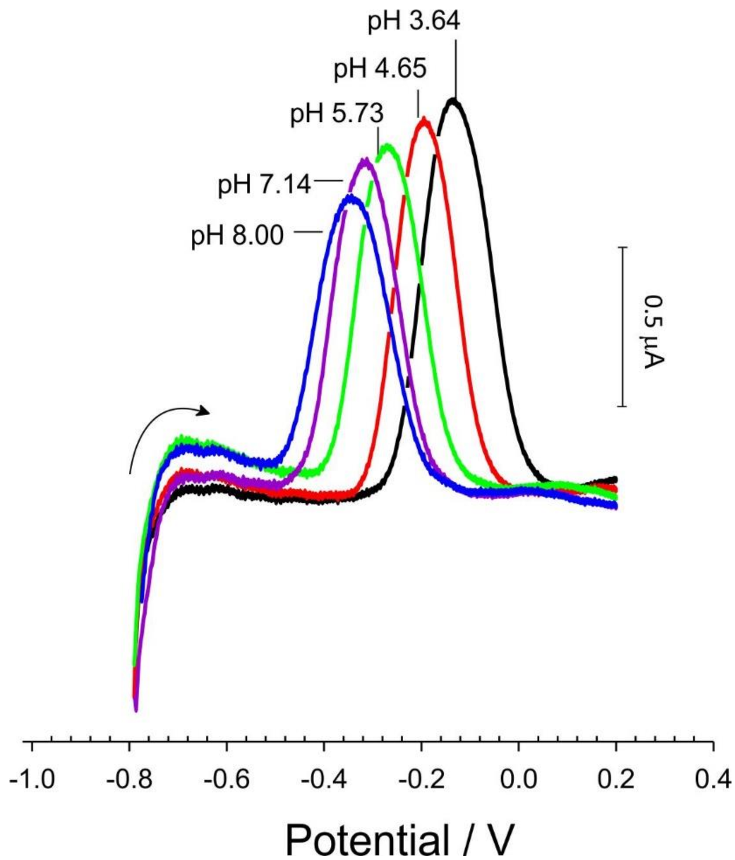


Figure 2. Square wave voltammograms detailing the flavin modified screen printed carbon electrode in buffers of varying pH without prior degassing of the solution.

The square wave scan commences at -0.8 V which has the effect of immediately reducing the immobilised flavin component (II→III). As the potential is swept towards positive values, the flavin is oxidised (III→II). The initial sharp rise in the current at observed at -0.8V reflects the fact the starting potential is on the cusp of the hydrogen evolution reaction. Starting the potential at more negative values (i.e. -1.0 V) could lead to the generation of bubble (particularly in acidic solution) which would comprise the voltammetric profile and could lead to changes in the interfacial pH leading to an erroneous response.

There are no other competing processes and it is clear that the peak process provides an easily identifiable signature. The peak position, as anticipated, moves with pH and exhibits near Nernstian behaviour with a shift of 57 mV/pH unit and is consistent with studies involving riboflavin immobilised on silver quantum dots [21].

The entire process was repeated a further two times – again cycling through a series of pH buffers and the square wave profile recorded. The influence of pH on the position of the flavin oxidation peak throughout the three consecutive cycles is detailed in **Figure 3A**. It can be seen that there is minimal drift in terms of the peak position from one series to another (less than 3 mV) and highlights the reversibility of the pH response of the immobilised redox group. The magnitude of the oxidation peak however was found to decrease with successive scans (**Figure 3B**) falling to 25% after 68 Scans and indicates a loss of the flavin from the surface.

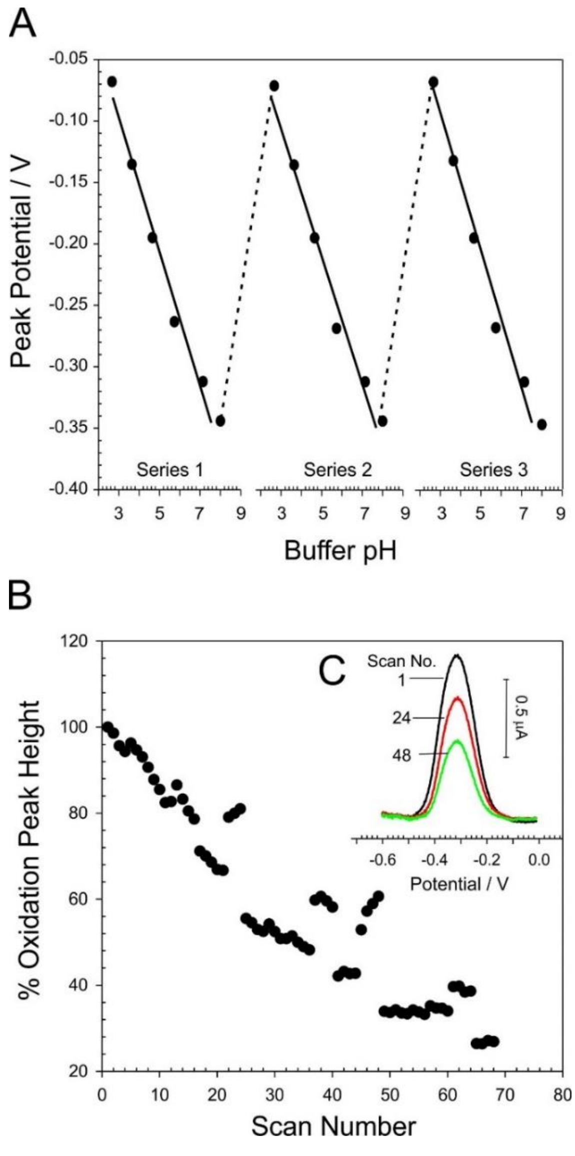


Figure 3. A) Influence of pH on the flavin oxidation peak position and B) Effect of repetitive scanning on the magnitude of the peak height.

The response diminished with increasing scan number and could be attributed to a number of factors. The steric hindrance of the short phenolic spacer which comprises the backbone of the polymer could result in the deposition of low molecular weight oligomers rather than an extensive network through which the redox groups would be anchored to the electrode surface. The response will also be influenced by the doping processes intrinsic to the electrolyte used during the film formation.

It must also be recognised that the composite nature of the SPE itself can be problematic in establishing the flavin polymer leading to isolated pockets of polymer rather than a continuous film. It should be noted that increasing the scan number during polymerisation in an attempt to increase the production of polymeric material, did not appreciably increase retention of the redox activity. Despite the decrease in response highlighted in Figure 3B, the shift in oxidation peak with pH remains consistent with minimal drift. A key advantage of exploiting the flavin redox process to measure pH relates to the fact that peak potentials lie within a region where there are few competing electrode processes. This is in contrast to the quinone systems where common electroactive components such as ascorbate or urate could be expected to create ambiguities through overlapping signals. In this case the peak separation between the flavin oxidation peak and ascorbate (at pH 7) is of the order of some 700 mV. It must be recognised however that nature of interference will depend on the sample matrix being investigated and it could be envisaged that other matrices where natural flavins are present may overlap.

While the responses observed are in line with Nernstian behaviour using a conventional Ag|AgCl half cell reference [21], it is clear that the peak position would be susceptible to fluctuations in the chloride activity were a solid state silver reference employed. In order to counter this issue, ferrocyanide was introduced as a pH insensitive redox probe. Rather than relying solely on the oxidation peak process of the flavin, the peak potential of the latter is measured relative to the oxidation peak associated with the oxidation of ferrocyanide. The expectation is that the latter will be insensitive to changing pH. It is however anticipated that the peak positions of both the flavin and ferrocyanide will be affected equally by fluctuations in chloride activity. As such the analytical signal could therefore be taken from the peak separation between the flavin and the ferrocyanide (acting as internal standard). Were this to be viable, then, in principle, the silver chloride component could be completely removed. It could be expected that this would greatly simplify the strip manufacturing processes and remove the cost overheads associated with silver ink.

Square wave voltammograms detailing the response of the SPE towards the flavin in the presence of ferrocyanide under varying pH conditions are detailed in **Figure 4**. The ferrocyanide oxidation peak is observed at +0.187 V and it can be seen that the position is relatively constant (± 6 mV). The response is near Nernstian: E_{pa} (Volts) = 0.057 (+/- 0.00355) pH – 0.167 (+/- 0.0178); N = 15; R^2 = 0.991 with the error estimates of the slope and intercept factoring in the variation in the ferrocyanide peak position. The error is based on an assessment of the 95% confidence interval.

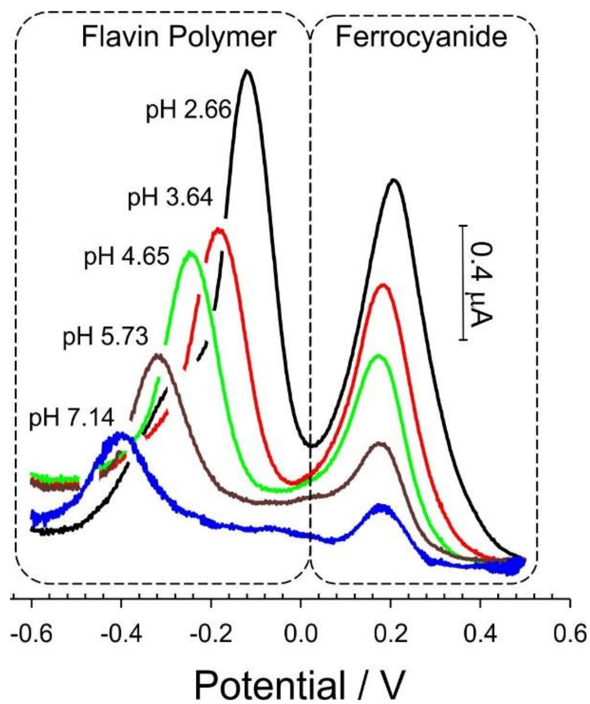


Figure 4. Square wave voltammograms detailing the influence of pH on the response of a flavin modified screen printed electrode with ferrocyanide as an internal standard.

A more quantitative exploration of the peak shift (based on the difference between the flavin and ferrocyanide oxidation peak processes is detailed in **Figure 5.** The response of the flavin oxidation peak to varying pH, measured against a conventional silver-silver chloride reference, is compared against the response calculated from the peak separation between flavin and ferrocyanide standard. It is important to note that in both cases – each point is the average of 3 measurements but the error, in all cases, is of the order of 1-2 mV and hence error bars are invisible under the scale employed. There is only a minor variation in the gradients between the two approaches with Nernstian type behaviour observed over the pH range studied and it is clear that the internal standard is a viable alternative to the conventional silver chloride reference.

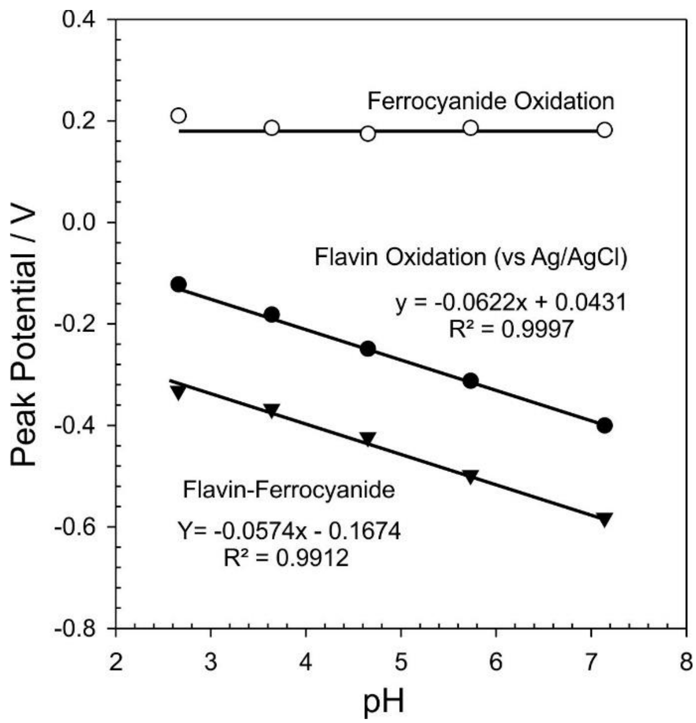


Figure 5. Square wave voltammograms detailing the influence of pH on the response of a flavin modified screen printed electrode with ferrocyanide as an internal standard.

Analytical Performance

The repeatability and inter electrode reproducibility of the measurements were assessed through comparing the response of the flavin modified electrodes in pH 6 buffer. Repeatability was determined through assessing the peak variation during 10 replicate measurements with the same electrode and same operator. Inter electrode reproducibility involved the same approach but used 5 individual electrodes (scans performed in triplicate) with each modified with the flavin (as discussed in the previous section). The %RSD for the repeatability and inter electrode comparisons was 1.03% and 1.65% respectively and highlights the robustness of the measurement and fabrication process.

The analytical performance of the flavin modified SPE was assessed through its application to the measurement of soy milk pH. The latter is increasingly used as an alternative to cows milk but it has garnered considerable attention as a key component within functional foods [26–32]. The health benefits of soy products in relation to low grade inflammation-related diseases are well documented [32]. Fermentation of the former with probiotic bacteria has been proffered as a means through which to increase the bioavailability and bio-effectiveness of soy constituents [26,32]. The fermentation processes are pH dependent and thus monitoring the latter is critical to regulating product specification (texture, flavour and nutritional composition) [26,28–31]. Soy milk products therefore possesses significant complexity (n-3 polyunsaturated fatty acids (PUFA), protein, isoflavones and a variable amino acid and carbohydrate profile) and thus was expected to provide a considerable challenge to the flavin/ferrocyanide sensor. The results from flavin modified SPE with and without the ferrocyanide internal standard are compared in **Table 1** along with the pH measured using a conventional glass pH probe. There is good agreement between the two methods and the commercial glass probe. The error is greater in the case of the internal standard and reflects the small variation in the peak position of the latter. Nevertheless, it is important to note that despite operating within a complex biological matrix – no interference or ambiguity in ascribing peak positions was encountered.

Milk Sample	Actual pH	Method	Mean pH	Error[a]
Soy Sweetened	6.66	Flavin Peak	6.75	± 0.076
		Flavin-Ferro	6.79	± 0.239
Soy Unsweetened	6.82	Flavin Peak	6.80	± 0.072
		Flavin-Ferro	6.88	± 0.233

Table 1. Response of the Flavin modified SPE in commercial soy milk samples
([a]Error estimated at a 95% Confidence Interval)

4.0 Conclusions

The flavin monomer has been shown to be capable of electropolymerisation on to a screen printed format and yields a redox signature which exhibits a Nernstian response over the range pH 3 - pH 8. The peaks are distinct and lie within a region of the potential window that is free from many of the interferences that impede acquisition of data from probes with more anodic potentials. The electrode response is shown to be consistent between pH sequences with minimal drift although the peak response has been found to diminish upon repetitive scanning. that while the loss in response is problematic for continuous monitoring applications, screen printed systems are normally designed for single shot use. It must be noted however, that the response was still measurable without any ambiguity even after 68 scans. The limitations of reference electrode component within conventional voltammetric sensing methodologies have long been recognised and the use of a ferrocyanide internal standard in this instance has been shown to provide a facile solution. The accuracy of the system – with and without a silver chloride reference has also been demonstrated. Overall, the system provides a simple, inexpensive platform for disposable pH sensing applications.

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Synthesis of intermediates: Charlotte M. Taylor

Supervision and characterisation of synthesis/products: Clare L. Lawrence, Robert B. Smith,

Supervision of overall project and compilation of manuscript: James Davis

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